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13. ABSTRACT *(Maximum 200 Words)*

This project focuses on the product of the HER2/Neu oncogene, a receptor tyrosine kinase that is amplified in 25-30% of human primary breast tumors. The overall goal of the project is to characterize the substrate specificity of the HER2/Neu kinase. In previous years, we expressed and purified HER2/Neu using the Sf9/baculovirus system. We then used peptide library technology to isolate novel peptide substrates for HER/Neu. One of them, AAEEIYAARRG, is the best synthetic peptide substrate reported to date for HER2/Neu. In the final year of the project, we produced several potential peptide-based inhibitors for HER2/Neu by replacing the tyrosine residue in the substrate peptide with the following amino acid analogs: (1) p-L-carboxy-Phe; (2) p-D-carboxy-Phe; (3) tetrafluoro-L-Phe; (4) tetrafluoro-D-Phe; (5) 3,5-diiodo-L-Phe. We produced an additional peptide inhibitor by introducing p-L-carboxy-Phe into another peptide isolated from the library (EDKVDYRMHRRG). The inhibitory potencies of these compounds against purified HER2/Neu were in the millimolar range; these values were too high for in vivo inhibition of HER2/Neu. Instead, we used a microinjection technique to measure the ability of the parent peptide AAEEIYAARRG to block EGF-induced membrane ruffling in a fibroblast model system. The peptide showed significant inhibition of the EGF effect.

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W Todd Miller 6/13/00

PI - Signature Date

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INTRODUCTION

Constitutive activation of signaling pathways involving tyrosine phosphorylation is believed to play a crucial role in human breast cancer. The receptor tyrosine kinase $p185^{HER2/neu}$ (Neu) is amplified in 20 to 30% of human primary breast cancers, and expression of Neu in tumor tissues is correlated with poor clinical prognosis (1). Transfection of NIH3T3 cells with the *neu* oncogene results in malignant transformation (2), and studies in transgenic mice that overexpress Neu indicate that breast tissue is particularly susceptible to the transforming properties of the *neu* oncogene (3). Neu is a 185 kilodalton protein that is structurally very similar to the EGF receptor: it has a cysteine-rich extracellular domain, a membrane-spanning region, and an intracellular tyrosine kinase domain (4). The mechanism by which Neu transmits its mitogenic signal is not completely clear, but the tyrosine kinase activity of the receptor plays a crucial role (1). The identities of the *in vivo* substrates for Neu are unknown, and the substrate specificity of the Neu tyrosine kinase has not previously been defined. The broad goal of this project is to obtain information about the substrate specificity of the Neu tyrosine kinase. This information will provide a clearer understanding of how this enzyme subverts the normal signalling pathways in cells to cause neoplastic growth. In the second phase of the project, we will attempt to design molecules that specifically interfere with the action of Neu tyrosine kinase *in vivo* and *in vitro*. The results of these investigations may suggest avenues for design of specific anticancer agents.

The specific objectives of our project are:

Objective 1. We will isolate the Neu tyrosine kinase from cultured breast cancer cells and study its specificity using combinatorial peptide libraries.

Objective 2. We will also study the specificity of Neu with a solid-phase tyrosine kinase assay.

Objective 3. Nonphosphorylatable tyrosine mimetics will be introduced into peptide sequences obtained from Objectives 1 and 2. These compounds will be tested as inhibitors of the Neu tyrosine kinase *in vitro*. Results will be compared for representatives of other classes of tyrosine kinases.

Objective 4. Active compounds from Objective 3 will be truncated stepwise from the N- and C-termini to determine the minimum peptide length necessary for specific inhibition of Neu. The resulting peptides will be tested as inhibitors of the proliferation of a breast cancer cell line, SKBR3, using a tritiated thymidine incorporation assay.

BODY OF REPORT

We have completed the studies as proposed for Technical Objectives 1, 2, and 3. We completed Technical Objective 4, but we needed to modify the study. In Objective 4, we found that the peptide-based inhibitors did not have a high inhibitory potency. Therefore, we modified Task 2 of Objective 4 (in vivo inhibition of HER2/Neu). Instead of testing the inhibitors in the breast cancer cell line SKBR3, we tested the parent peptide for inhibition of EGF receptor in a rat fibroblast model system.

Objective 1. Studying Neu specificity with peptide libraries.

Task 1. Purification of Neu. As reported in the June 1999 progress report, we produced the HER2/Neu kinase using the Sf9/baculovirus expression system. We carried out a partial purification of the enzyme by FPLC using anion chromatography on HiLoad Q Sepharose. This preparation of Neu reproducibly resulted in high yields of Neu, and the enzyme was active and suitable for our further experiments. We carried out experiments to characterize its enzymatic activity.

Task 2. Preparation of peptide libraries. Our synthetic strategy for preparing peptide libraries was described in the June 1998 Progress report. Because we established that residues N-terminal to tyrosine are most important as determinants for Neu phosphorylation, we focused on a library of 20^3 (= 8,000) peptides in which residues both N-terminal to tyrosine have been randomized:

Library I: Ala-Ala-**X**-**X**-**X**-Tyr-Ala-Ala-Arg-Arg-Gly

(where **X** represents an equimolar mixture of all 20 common amino acids at a given position). Analysis of the library by Edman degradation, amino acid analysis, and mass spectrometry indicated that it contained all of the desired sequences in relatively uniform concentrations.

Task 3. Phosphorylation of peptide library by Neu. The details of these experiments are given in our June 1999 Progress Report. Neu phosphorylation of peptide library I was carried out using procedures we established previously for PDGF receptor phosphorylation (5). Briefly, Neu reactions with the library were carried out in the presence of [γ-^{32}P]ATP. Phosphorylated peptides were isolated using a ferric iminodiacetic acid (IDA) column (6), then separated on a narrowbore C18 HPLC column. This experiment resulted in the identification of an optimal substrate sequence for Neu: Ala-Ala-Glu-Glu-Ile-Tyr-Ala-Ala-Arg-Arg-Gly (Table 1).

Task 4. Phosphorylation kinetics of peptide containing optimal sequence. As reported in the June 1999 Progress Report, we prepared an individual peptide containing the sequence isolated from Peptide Library I (Ala-Ala-Glu-Glu-Ile-Tyr-Ala-Ala-Arg-Arg-Gly). This peptide was synthesized by standard FMOC procedures, purified by reverse-phase HPLC on a C18 column, and characterized by MALDI-TOF mass spectrometry. Kinase reactions were performed in microtiter plates using a continuous spectrophotometric assay (7). Kinetic parameters (K_m and V_{max}) were calculated by varying peptide substrate concentrations and measuring reaction velocity at 15 second time intervals. Statistical analyses were carried out by fitting initial velocity rates into the Michaelis-Menten equation using MacCurve Fit. Peptide 1 (AAEEIYAARRG) had a V_{max} of 2.4 +/- 0.05 μmol/min/mg and a K_m value of 158.11 +/- 32.2μM (Table 1) These measurements confirm the results of the peptide library study; this peptide is the best sequence reported to date for phosphorylation by Neu.

Objective 2. Studying Neu specificity with a solid-phase kinase assay.

Task 1. Preparing Neu for the solid-phase kinase assay. As described above, Neu was prepared by infecting Sf9 insect cells with baculovirus encoding the V664E Neu (t-Neu) receptor. t-Neu receptor was partially purified by anion chromatography using HiLoad Q Sepharose.

Task 2. Probing solid-phase library with Neu. As described in our June 1999 Progress Report, we used a solid-phase peptide library strategy to screen for additional Neu substrates. These experiments were carried out in collaboration with Dr. Peter Nestler of Cold Spring Harbor Laboratory. Dr. Nestler produced for us a peptide library containing six degenerate positions. This library, Peptide library II, (EDXXXYXXXG, where X represents a degenerate position excluding tyrosine and cysteine) was synthesized using Fmoc chemistry. Neu phosphorylation of peptide library II was carried out in 500 μL volumes with 10mg peptide-beads in kinase reaction buffer (100mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 4mM $MnCl_2$, 100μM Na_3VO_4, 1mg/mL bovine serum albumin, and 200μM [γ-^{32}P]ATP). The reaction was incubated at 30 degrees for 6 hours with constant stirring in reaction vials. The peptide-beads were then washed two times in 1mL volumes alternately with 50mM Tris pH 7.4, 1mM ATP and with 50mM Tris pH 7.4, 1mM ATP, 0.5% SDS. This was followed by washing alternately with 0.5% HCl and 0.5% phosphoric acid for a total of ten washes. The peptide-beads were then washed twice with deionized water. Our initial control experiments using peptides with defined sequences confirmed that Neu was capable of phosphorylating immobilized peptides in this form of the solid-phase kinase assay (data not shown). To identify substrate sequences from the library, the large-scale reaction was analyzed as described below under Task 3.

Task 3. Determining the sequences of Neu substrates. After reaction with Neu, peptide-beads were then resuspended in 0.5% gelatin (Sigma) and spread evenly on glass plates (8). In a dark room, autoradiography emulsion (Kodak) was spread on the glass plates and allowed to develop for 2 weeks at the temperature of –70°C. Darkly stained beads were extracted using fine forceps and placed in individual glass tubes. The peptide on each bead was identified by Edman degradation. The individual peptides isolated by this solid-phase kinase assay of Neu are presented in Table 1. Most of these sequences contain previously unidentified motifs for phosphorylation by Neu. Interestingly, some of them contain amino acid sequences which bear some resemblance to the sites of autophosphorylation on Neu.

Task 4. Synthesize individual peptides corresponding to optimal sequences; measure kinetics of phosphorylation by Neu. These experiments are described in detail in the June 1999 Progress Report. Four individual peptides corresponding to the sequences of phosphorylated peptides from peptide library II were synthesized using Fmoc protocols and used for kinetic analyses. The results are presented in Table 1. Of the five peptides (i.e., one peptide from Objective 1 and four from Objective 2), peptide 1 and 4 are the best substrates for Neu receptor kinase as seen in their V_{max}/K_m values of 15.1 x 10^{-3} and 9.6 x 10^{-3}, respectively. As a control for Neu phosphorylation, a peptide with alanines surrounding the phosphoaccepting tyrosine (AAAAAYAARRG) was used. This peptide had a V_{max} of 1.7 +/- 0.1μmol/min/mg and a K_m value of 1703.5 +/- 245.3μM (Table 1).

Objective 3. Peptide Inhibitors of Neu.

Task 1. Synthesize, purify, and characterize peptide inhibitors of Neu. We described an initial inhibitor in the June 1999 Progress Report. We have now synthesized 6 potential peptide-based inhibitors of HER2/Neu tyrosine kinase (Figure 1). We generated Neu inhibitors by replacing the phosphorylatable tyrosine in peptides 1 and 4 (see above). The tyrosine

mimetic that we chose for our initial generation of inhibitors was *para*-carboxylphenylalanine (*p*-COOH-F), which has been used successfully by Dr. John McMurray and his collaborators at the M.D. Anderson Cancer Center as a tyrosine mimetic for Src inhibitors (J. McMurray, personal communication). The peptides were synthesized by standard FMOC procedures using a racemic D,L-mixture of FMOC-*p*-COOH-F. The structures of the inhibitors are given in Figure 1. Purification of peptides containing D and L enantiomers of *para*-carboxylphenylalanine (*p*-COOH-F) was carried out using reversed-phased HPLC with an analytical HPLC column. The peptides were purified using a 30 minute gradient from 5 to 75% acetonitrile in 0.1% TFA. For peptide AAEEI-(D,L) *p*-COOH-F-AARRG, absorbance peaks were observed at 21.5 minutes and 22 minutes. For peptide EDKVD-(D,L) *p*-COOH-F-RMHRRG, absorbance was observed at 24.3 minutes. Mass spectral analysis confirmed these peaks to be the correct peptides. To better resolve the D and L diastereomers of these and other inhibitors, we employed an HPLC solvent system consisting of 0-50% acetonitrile in 200mM $NaClO_4$, 25mM NaH_2PO_4, pH 2.5 (9).

To identify which peak from the separations of inhibitors corresponded to which diastereomers, the HPLC peaks were analyzed by leucine aminopeptidase digestion (10). Leucine aminopeptidase was preactivated by incubation at 37°C for 1 hour in 100mM Tris pH 8.5, 20mM MnCl2. 100μg of peptide from each peak was added to the preactivated aminopeptidase and digested at 37°C for 3 hours. The digested peptides were desalted and subjected to mass spectral analysis. For example, for the peptide with the sequence, AAEEI-(D,L) *p*-COOH-F-AARRG, the first peak (21.5') showed a component with the mass of 387.3, corresponding to residues RRG which has a mass of 388.5. There were no other components with greater mass. The second peak (22') showed a component with the mass of 722, corresponding to residues (D)*p*-COOH-F-AARRG which has a mass of 721.8. As leucine aminopeptidase recognizes only L-amino acids, it was concluded that the first peak corresponded to the peptide containing the L enantiomer of *p*-COOH-F (inhibitor L-1) and the second peak contained the D enantiomer.

We produced additional peptide-based inhibitors by introducing the following non-phosphorylatable tyrosine surrogates into Peptide 1: tetrafluoro-L-Tyr, tetrafluoro-D Tyr, and 3,5-diiodotyrosine. Each of these inhibitors has been used as a tyrosine mimetic in the generation of inhibitors for other tyrosine kinases (10,11). The synthetic strategy we used was similar to that described above for p-carboxy-Phe. Tetrafluro-D,L-Tyr was the kind gift of Dennis McNamara (Parke-Davis), and 3,5-diiodoTyr was commercially available. The peptides were synthesized using an FMOC strategy, purified by HPLC, and characterized as described above.

Task 2. Test peptides as inhibitors of Neu in vitro. The inhibition constant K_i for all peptide inhibitors was determined using the continuous spectrophotometric assay (7), with varying amounts of inhibitor (50-2000μM) and two fixed concentrations of peptide substrate (300 and 600μM). K_i values were determined graphically according to the method of Dixon (12). The values are presented in Figure 1. Thus, the best inhibitor of Neu in this set is the L enantiomer of tetrafluoroTyr, with a K_i of 0.4 mM. The millimolar K_i values for the inhibitors contrast with the K_m value of 158 μM for Peptide 1, the peptide from which the inhibitors were derived. These results indicate that the sidechain of tyrosine is a major determinant for substrate recognition by the HER2/Neu tyrosine kinase.

Task 3: Analyze pattern of inhibition for most potent inhibitors; test inhibitors against other protein kinases. The inhibition constants K_i for all peptide inhibitors were in the millimolar range (Figure 1). In this experiment, we analyzed the pattern of inhibition for the L-tetrafluoroTyr peptide by varying the peptide substrate concentration in the presence of various concentrations of the inhibitor. The results were consistent with the mechanism of inhibition being competitive with respect to peptide substrate. We also carried out similar experiments using the tyrosine kinase Hck, a member of the Src family of kinases. These experiments indicated that the potency of the peptide inhibitor towards Hck was similar to the potency observed towards Neu. For this reason, and given the relatively weak inhibitory potency of the peptide inhibitors, the inhibitors in their present form are unlikely to serve as useful inhibitors of Neu kinase in vivo.

Objective 4. In vivo inhibition of Neu. As described above under Task 3, the peptide-based inhibitors that we produced had a relatively weak potency and did not appear to be selective for Neu. For these reasons, we elected not to pursue in vivo inhibition of Neu in the SKBR3 cells. Instead, we took advantage of the fact that the parent peptide (Peptide 1 in Table 1) displayed a relatively low K_m for Neu (158 μM). In collaboration with Prof. Dafna Bar-Sagi (Dept. of Microbiology, Stony Brook), we introduced Peptide 1 into rat embryo fibroblast cells and assayed the ability of this peptide to block an EGF-mediated response. The rationale for these studies was that HER2/Neu and EGF receptor are closely related proteins, and molecules that interfere with the function of one kinase are likely to interfere with the other as well.

Task 1. Establish tritiated thymidine incorporation assay. We did not use this assay.

Task 2. Test peptides as inhibitors of Neu in vivo. Dr. Bar-Sagi's group has established a fibroblast model system to study EGF and other growth factor signalling using rat embryo fibroblast REF52 cells (13). In these experiments, REF52 cells are plated onto glass cover slips and cultured in DMEM supplemented with FBS (10%). The cells are grown to confluence, then placed in DMEM with 0.5% FBS for 24 hours prior to microinjection. The cells were treated with EGF, then microinjected with a solution of Peptide 1 (or PBS control). Cells were then fixed and observed by light microscopy (Figure 2). In these experiments, the cells respond to EGF by activating multiple signalling pathways, including the MAP kinase pathway. One of the characteristic morphological changes that occurs is membrane ruffling, a result of actin cytoskeletal reorganization (13). Peptide 1, but not a PBS microinjection control, was able to partially block the membrane ruffling induced by EGF in the REF52 cells (Figure 2). This presumably occurs by a mechanism involving substrate binding to the kinase domain of EGF receptor, blocking access to other key signalling intermediates. Thus, we demonstrated the ability of the HER2/Neu substrate peptide to interfere with a related HER family member in vivo.

FIGURE LEGENDS

Table 1. Substrate sequences for Neu identified in this study, along with kinetic parameters for phosphorylation.

Figure 1. Structures of peptide inhibitors and inhibitory constants.

Figure 2. Inhibition of EGF-induced membrane ruffling in REF52 cells by Peptide 1. Quiescent REF52 cells were microinjected with PBS control or Peptide 1 (200 μM) as described in the text. Membrane ruffling was observed by light microscopy; membrane ruffles are indicated by arrows.

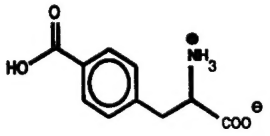
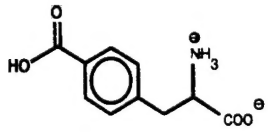
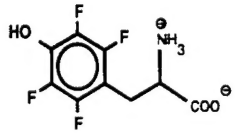
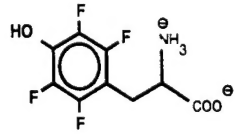
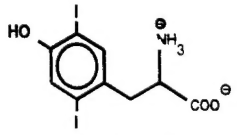
Table 1. Neu substrate sequences identified from Peptide libraries I and II, and kinetic analyses of individual peptides

Peptide Sequence	Library used	V_{max} (μmol/min/mg)	K_m (μM)	V_{max}/K_m (10^{-3})
1. AAEEIYAARRG	I	2.4 +/- 0.05	158.11 +/- 32.2	15.1
2. EDGPIYQMARRG	II	n.d.	n.d.	0.02*
3. EDFAMYLNSRRG	II	2.6 +/- 0.02	382.6 +/- 38.1	6.8
4. EDKVDYRMHRRG	II	2.35 +/- 0.23	245.2 +/- 28.7	9.6
5. EDFQKYKMLRRG	II	5.9 +/- 0.07	1401.3 +/- 233.1	4.2
6. AAAAAYAARRG	control	1.7 +/- 0.1	1703.5 +/- 245.3	1.0

Sequences isolated from Peptide library I (Technical Objective 1) and Peptide library II (using the assay described in Technical Objective 2). Underlined residues correspond to the degenerate positions of the two peptide libraries. Peptides corresponding to the sequences identified from the screening of peptide libraries I and II were synthesized. Kinetic analyses of Neu receptor kinase were carried out using the continuous spectrophotometric assay (5). Peptide 6 contains a control sequence. *Phosphorylation rates for peptide 2 were too low to measure kinetic parameters using this assay. The V_{max}/K_m value was determined graphically by using substrate concentrations that were much lower than K_m.

Figure 1. Peptide-based inhibitors of HER2/Neu.
The tyrosine residues in AAEEIYAARRG or EDKVDYRMHRRG were replaced with non-phosphorylatable tyrosine mimetics.

AAEEI-[X]-AARRG

Structure: [X] =	K_i
 4-carboxy-L-Phe	**1.6 mM**
 4-carboxy-D-Phe	**4.3 mM**
 tetrafluoro-L-Tyr	**0.4 mM**
 tetrafluoro-D-Tyr	**1.5 mM**
 3,5-diiodo-L-Tyr	**1.5 mM**

EDKVD-[X]-RMHRRG

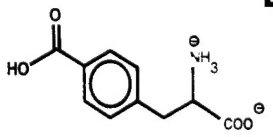
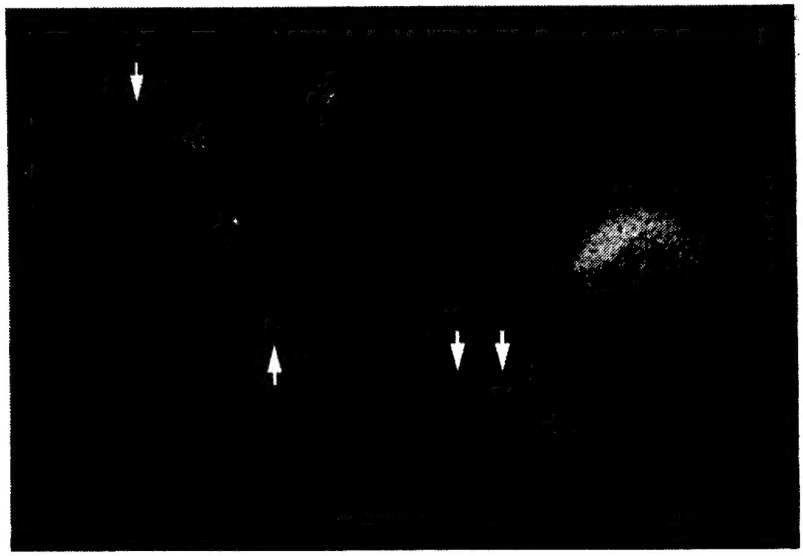
 4-carboxy-L-Phe	**7.8 mM**

Figure 2. Peptide 1 interferes with EGF-induced membrane ruffling in REF52 fibroblasts.

The arrowheads indicate sites of membrane ruffling.

A. microinjection: buffer control



B. microinjection: Peptide 1

KEY RESEARCH ACCOMPLISHMENTS.

- Produced full-length, active form of Neu
- Tested substrate specificity of Neu using two combinatorial library techniques
- Identified five novel substrate sequences for Neu. Confirmed using individual peptides. One peptide is the best available substrate for Neu kinase.
- Synthesized and tested peptide inhibitors of Neu.
- Demonstrated the feasibility of in vivo inhibition of HER family members using synthetic peptides.

REPORTABLE OUTCOMES.

1. A manuscript describing the results obtained in Technical Objectives 1 and 2 has been submitted for publication.

2. A poster describing the work in Technical Objectives 1-3 was presented at the DOD Breast Cancer Program Era of Hope meeting in June 2000 (Poster # CC-1).

3. The results described above formed a major part of the Ph.D. thesis of Perry M. Chan, a student in the Program in Molecular and Cellular Biology at SUNY Stony Brook. Dr. Chan received his Ph.D. in August 1999 and is now pursuing postdoctoral work at the University of Toronto,

CONCLUSIONS

The overall goal of this project is to characterize the substrate specificity of the Neu tyrosine kinase. We have isolated the Neu protein from Sf9 insect cells using a baculovirus expression vector. The Neu that we have prepared from these sources is active. We report here the results of experiments conducted using combinatorial peptide libraries, including a solid-phase kinase assay. We have isolated five novel sequences from the libraries that are phosphorylated efficiently by Neu: AAEEIYAARRG, EDGPIYQMARRG, EDFAMYLNSRRG, EDKVDYRMHRRG, and EDFQKYKMLRRG, where the underlined sequence represents the randomized positions. We confirmed that these sequences represent Neu substrates using individually-synthesized peptides, and determined the kinetic parameters for phosphorylation by Neu. One of them (Peptide 1; AAEEIYAARRG) is the best synthetic peptide substrate reported to date for Neu. We synthesized potential peptide-based inhibitors of Neu by replacing the phosphorylatable tyrosine in this peptide and another peptide with various non-phosphorylatable tyrosine mimetics. The inhibitory potency of these peptides toward purified Neu is somewhat low, and they do not appear to be selective for HER2/Neu. Nonetheless, we demonstrated the feasibility of using the peptides to inhibit a HER family member by microinjecting peptide 1 into REF52 fibroblasts.

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INFORMATION FOR FINAL REPORT

1. Bibliography

Chan, P.M., Nestler, H.P., and Miller, W.T. Investigating the substrate specificity of the HER2/Neu tyrosine kinase using peptide libraries. Submitted for publication.

Chan, P.M., Nestler, H.P., and Miller, W.T. Investigating the substrate specificity of the HER2/Neu tyrosine kinase using peptide libraries. Abstract presented at the Department of Defense Breast Cancer Program Era of Hope Meeting, Atlanta, GA, June 2000.

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